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The overall goal of this contract effort was to determine the effectiveness of microencapsulated vaccines compared to the conventional vaccines in inducing protective immune responses against challenge with the pathogens Venezuelan equine encephalitis (VEE) virus and *Bacillus anthracis*. The microsphere vaccines were formulated of the biodegradable and biocompatible co-polymer poly(DL-lactide-co-glycolide) (DL-PLG). Formalin-fixed and/or ^{60}Co-inactivated TC-83 VEE virus or protective antigen (PA) of *B. anthracis* was encapsulated in DL-PLG by an emulsion-based process to yield microspheres of approximately 1 to 10 μm in diameter and containing approximately 0.8% by weight antigen. Mice immunized by the systemic route with antigen encapsulated in microspheres composed of equal amounts of lactide and glycolide had higher protective immune responses than animals immunized with antigen alone. Vaccine prepared with formalin-fixed virus induced higher responses than untreated virus. Microencapsulated VEE prepared with the solvent methylene chloride compared to ethyl acetate induced higher levels of ELISA antibody activity, neutralization titers, and protection against systemic challenge. However, this relationship depended on the dose of vaccine. Similarly, microencapsulated PA induced higher responses than free antigen and induced the greatest protection against aerosol challenge when given to guinea pigs. Finally, mucosal, especially intratracheal, immunization with microencapsulated VEE induced systemic and mucosal responses and protection against aerosol challenge.

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Suzanne M. Michalek 9/20/95

PI - Signature Date

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INTRODUCTION

Numerous infectious agents are acquired in nature through aerosol exposure. Included in this group are members of the arboviruses and *Bacillus anthracis* which are pathogenic for humans. Although vaccines have been produced and systematically tested for a few of the arboviruses, there is evidence that the form of the vaccine determines the extent to which protection against aerosol exposure is provided. A case in point are the Venezuelan equine encephalitis (VEE) virus vaccines TC-83 and C-84. Immunization of hamsters with the live attenuated strain TC-83 confers protection against both aerosol and subcutaneous challenge with virulent VEE, while multiple immunizations with the formaldehyde-killed form, C-84, is only protective when challenge is performed by the subcutaneous route (1). Thus, the inactivated vaccine for this virus suffers from both poor immunogenicity and an inability to induce protection against viral penetration through the respiratory tract, even when circulating antibodies are induced.

The successful protection against pulmonary exposure afforded by the live-attenuated VEE strain demonstrates that vaccines of this type can be extremely effective. However, the production and testing of live virus vaccines is exceedingly time consuming and administratively difficult. An approach which could overcome the deficiencies in the immunogenicity of inactivated whole virus vaccines, particularly with respect to antibodies in the respiratory tract, would thus make the development of vaccines to a wide range of airborne viral and microbial pathogens substantially easier to accomplish.

Antibodies which are present within the respiratory tract originate from two different sources. The bronchioli and alveoli predominately contain IgG which is passively derived from the intravascular pool (2,3). In contrast, secretory IgA (S-IgA) predominates in mucus which bathes the nasopharynx and bronchial tree (4-6). The bulk of bronchial IgA is locally produced (2) by IgA plasma cells which line the lamina propria of the airway wall and are particularly concentrated about the bronchial glands (4). Importantly, numerous investigations have shown that S-IgA levels in humans (7,8) and mice (9,10) correlate with protection against pulmonary viral challenge to a significantly greater extent than do circulating antibody titers.

Unlike the blood circulation, the induction of antibodies at the mucosa of the upper respiratory tract is not commonly achieved through parenteral immunization with inactivated vaccines. S-IgA antibodies are, in general, only induced through direct immunization of mucosal-associated lymphoid tissues (MALT), such as those found in the gastrointestinal tract and bronchial tree (11-13). One possible reason for the inability of the C-84 VEE virus vaccine to protect against aerosol exposure may be the failure of this parenterally administered vaccine to induce S-IgA antibodies. However, simple mucosal application of C-84 is not an effective strategy because of antigenic degradation at these surfaces, and inefficient adsorption into the MALT. Thus, immunization through MALT with an inactivated vaccine to protect against the aerosol spread of infectious agents requires a method of vaccine delivery which will protect the antigen from degradation in the gut or respiratory tract lumen, enhance its uptake into the MALT and potentiate the subsequent immune response. One vaccine delivery method which has the potential to provide these characteristics is microencapsulation.

Microencapsulation involves the coating of a bioactive agent, such as vaccine antigens, in a protective wall material which is generally polymeric in nature. The microsphere product is a free-flowing powder of spherical particles which can be produced across a size range from ≤ 1 μm to as large as 3 mm in diameter. The particular system investigated in the studies reported here involves the use of DL-PLG copolymers (14-16). DL-PLG is in the class of copolymers from which resorbable sutures, resorbable surgical clips and controlled-release drug microspheres and implants are made (17). These biocompatible polyesters are approved for and have a 30 year history of safe use in humans. After introduction into the body, DL-PLG induces only a mild inflammatory response and biodegrades through hydrolysis of ester linkages to yield the normal body constituents lactic and glycolic acids (18,19). Furthermore, the rate at which DL-PLG biodegrades is a function of the ratio of lactide to glycolide in the copolymer (20), thus determining the time after administration when vaccine antigen release initiates, and the subsequent rate of release (16,21).

Studies have shown that the systemic injection of staphylococcal enterotoxin B (SEB) toxoid (14-16,22-24), influenza vaccine (25,26), simian immunodeficiency virus (SIV) vaccine (23) or ovalbumin (27,28) encapsulated in 1-10 μm DL-PLG microspheres results in a strongly potentiated antibody response. In the case of SEB toxoid, mice immunized with 50 μg of vaccine antigen in microspheres mounted a neutralizing plasma anti-toxin response which was equivalent in level and duration to that induced by the same dose of toxoid in complete Freund's adjuvant (CFA), but without an inflammatory response (14,28). Similar immunopotentiation has been obtained in

rhesus macaques immunized by intramuscular injection of microencapsulated SEB toxoid or whole formalin-inactivated SIV (23). Furthermore, mixtures of microspheres with varying sizes and/or lactide to glycolide ratios have been used to deliver multiple discrete releases of vaccine antigen following a single injection (16). Thus, this system allows the formulation of a vaccine which can be used to deliver primary and timed booster immunizations in a single administration.

DL-PLG microspheres have also been found to be an effective vehicle for mucosal immunization via the oral (15,16,21,24) and intratracheal (23,24) routes. This activity is attributable to the protection against nonspecific and specific proteolytic degradation provided by the encapsulation, as well as the enhanced and targeted delivery of the intact vaccine into the MALT. This adsorption of microspheres into the MALT from the lumen of the gut or respiratory tract is through a phagocytic-like mechanism restricted to particles of ≤ 10 μm in diameter (15,16,22).

The overall goal of this contract effort was to determine the effectiveness of microencapsulated vaccines compared to the conventional vaccine form in inducing protective immune responses. The vaccine antigens studies were VEE and the protective antigen (PA) of *B. anthracis*. The objectives of the studies were to investigate the potential of; 1) microencapsulated vaccines in potentiating immune responses to the VEE or PA antigen, 2) a single administration of a multiple release microsphere vaccine containing VEE antigen in inducing long term responses, and 3) mucosal immunization with microencapsulated VEE vaccine in inducing protective responses. Included in the later investigations was oral and intratracheal immunization both singly and as boosters following systemic priming.

MATERIALS AND METHODS

Mice. Specific pathogen-free BALB/c mice of mixed sexes were used throughout these experiments. They were bred and maintained in our barrier facilities at the University of Alabama at Birmingham. They were allowed food and water *ad libitum* and were entered into experimental protocols at 8-12 weeks of age. All animal studies were conducted in compliance with the principles stated in the "Guide for Care and Use of Laboratory Animals", of the Institute of Animal Resources, National Research Council (NIH publication 86-23, revised 1985). All animal facilities at UAB are under the direction of full-time veterinarians and are fully accredited by the American Association for Accreditation of Laboratory Animal Care. UAB complies with the NIH policy on animal welfare (assurance number A3255-01), the Animal Welfare Act and all other applicable federal, state and local laws.

Vaccine antigens. The VEE virus vaccine used in these studies was the attenuated vaccine strain TC-83 which was grown in BHK-21 cells in the laboratories of Dr. Jonathan F. Smith in the Virology Division, USAMRIID, Ft. Detrick, MD. After a single passage to expand the infectious stock of virus, monolayers of cells in 850 cm^2 roller bottles were infected at an MOI of 1 and cultured for 24 hr. The culture supernatants were harvested and clarified by centrifugation (10,000 x g) for 20 min at 4°C prior to bringing the solution to a concentration of 7% polyethylene glycol and 0.5 M NaCl. Virus was allowed to precipitate from this solution overnight at 4°C, after which the precipitate was packed by centrifugation. Following resuspension in a minimal volume of H_2O, the virus was isolated by banding in a 20 to 60 wt/wt% sucrose gradient. Fractions containing virus free of contaminants were selected on the basis of SDS polyacrylamide electrophoresis gels stained with coomasie brilliant blue. A portion of the purified virus was fixed by suspending the pelleted virus in phosphate-buffered saline (PBS) and formaldehyde (37%) to a final concentration of 0.025% and incubating overnight at room temperature. Formalin-fixed and untreated pelleted virus were inactivated by ^{60}Co-irradiation. The PA of *B. anthracis* was provided by Dr. Arthur M. Friedlander in the Bacteriology Division, USAMRIID, Ft. Detrick, MD.

Microencapsulation of vaccine antigens and characterization of the microspheres. The ^{60}Co-inactivated VEE virus or PA of *B. anthracis* was suspended in PBS to a concentration of approximately 30 mg/ml protein and the exact protein concentration was determined using the BCA assay (Pierce Chemical Company, Rockford, IL). The vaccine antigen was microencapsulated by an emulsion-based process (14) in which the excipient solvent, either methylene chloride or ethyl acetate, was removed by extraction. The surface morphology of each batch of microspheres was examined from photomicrographs obtained by scanning electron microscopy. This confirmed that a smooth surface of continuous polymeric coating had been obtained in each case. The vaccine content (core loading) was determined by dissolving a sample of the microspheres in 1.0 M sodium hydroxide, determining the amount of antigen obtained, and calculating the percent antigen by weight. Size distributions of each batch of microspheres were determined using a particle size analyzer (Malvern Instruments,, Malvern, United Kingdom). The results of these analyses were plotted to show the number fraction of the microspheres in each lot having given diameters and were calculated and plotted to show the number fraction having a given volume. *In vitro*

vaccine release kinetics were determined by placing a sample of the microspheres in a receiving fluid consisting of 0.5 M phosphate (pH 6.8), and the buffer was exchanged at 6 hr, 24 hr and every 24 hr thereafter until termination of the study. The amount of protein in the receiving fluids was quantified and related to the total protein in the sample of microspheres to determine the cumulative percent antigen release as a function of time.

Immunizations. Microencapsulated and non-microencapsulated antigens were suspended in PBS just prior to administration. Mice were immunized by subcutaneous (SC) injection of the microencapsulated or free antigen in a total volume of 0.5 ml in the nape of the neck. Mice immunized by the oral route were given the vaccine in a total volume of 0.5 ml by gastric intubation using a blunt feeding needle. Bronchopulmonary immunization by intratracheal (IT) instillation was done on mice anesthetized by intraperitoneal (IP) injection (0.2 ml) of a 1/10 dilution of a solution consisting of 100 mg of ketamine per ml and 1.5 mg of xylazine per ml. Mice were suspended by their lower incisors from a dissecting board maintained at a 45° angle. The vaccine was administered in 50 µl with the aid of a blunt tip feeding needle inserted through the glottis.

Collection of biological fluids. Blood was collected from the retro-orbital plexus of anesthetized mice using calibrated heparinized capillary tubes. Plasma samples were collected following centrifugation and stored individually or as group pools at -70°C until assayed. Fresh fecal pellets (4-6/mouse) were collected from each mouse in separate tubes. To each tube was added borate-buffered saline (BBS) containing 0.01% sodium azide at a ratio of 1/10 (wt/vol). The tubes were vortexed, allowed to settle (15 min) and vortexed again. After repeating this procedure, the samples were centrifuged (13,000 x g, 10 min, 4°C) and the supernatants were collected and stored at -70°C until assayed for antibody activity. Gut wash and saliva samples were collected and processed as routinely performed. Briefly, mice were given a high-salt lavage solution (0.5 ml) by gastric intubation four times at 15 min intervals. Fifteen min after the last treatment, mice were given by IP injection, sodium pentobarbital (1 mg/0.2 ml sterile saline) followed by pilocarpine (0.1 mg/0.2 ml sterile saline) 15 min later. The discharge intestinal contents were collected into petri dishes containing 5 ml of 50 mM EDTA with 0.1 mg/ml soybean trypsin inhibitor. The intestinal material was thoroughly mixed and centrifuged at 650 x g for 10 min to remove solids. The supernatant was then treated with 30 µl of 100 mM phenylmethylsulfonyl fluoride, clarified by centrifugation (27,000 x g, 20 min, 4°C) and 20 µl of a solution of 1% sodium azide and 1% fetal bovine serum was added. Saliva (approximately 250 µl/mouse) was collected by capillary action into Pasteur pipettes concurrently with intestinal discharge and clarified by centrifugation. Mice were then killed and BAW fluids were obtained by flushing the lungs a total of three times with 1 ml RPMI 1640 and clarified by centrifugation. Samples of gut and BAW fluids and saliva were stored individually and as group pools at -70°C until assayed.

Antibody analysis. An enzyme-linked immunosorbent assays (ELISA) was used to determine the levels of IgM, IgG and IgA antibody activity in plasma and secretion samples. Rigid 96-well assay plates (Pro-Bind, Becton Dickinson, Lincoln Park, NJ) were coated overnight with freeze fractured VEE virus or PA at 1 µg/ml in borate-buffered saline (BBS). All washing steps employed PBS containing 0.05% Tween 20 (PBS-Tween) and the diluent for all samples of reagents was PBS-Tween with 1% BSA. After blocking, serial 2-fold dilutions of the plasma samples, in triplicate, were added and incubated at 25°C for 6 hr. Antigen-specific antibodies were detected by sequential incubation with optimal dilutions of biotinylated goat anti-mouse IgM, IgG or IgA heavy chain-specific antibody overnight at 4°C (Southern Biotechnology Associates, Birmingham, AL), horseradish peroxidase-streptavidin for 2 hr at 25°C and the substrate 2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonic acid) at 0.3 mg/ml in pH 4.0 citrate buffer containing 0.0003% H_2O_2. The developed color was read after 15 min at 405 nm on a model EL312 kinetics reader (Bio-Tech Instruments, Inc., Winooski, VT) and the results presented as the reciprocal of the greatest sample dilution producing a signal significantly greater than that of the group-matched prebleed at the same dilution (endpoint titration).

Neutralization of VEE virus infectivity. Selected plasma samples from mice immunized with microencapsulated and non-microencapsulated VEE virus vaccine were tested for neutralization of VEE virus infectivity. These *in vitro* assays were performed in the laboratories of Dr. Jonathan F. Smith, USAMRIID, Ft. Detrick, MD.

Animal challenge. All animal challenge studies were performed at USAMRIID, Ft. Detrick, MD. For studies involving systemic challenge with VEE, mice were given by IP inoculation, $5x10^4$ pfu of the virulent V3000 strain of VEE virus in a volume of 0.2 ml on experimental day 80. Mice were monitored daily and the number of deaths recorded. In other studies involving aerosol challenge, mice were exposed for 10 min to an infectious aerosol of VEE virus strain V3000 generated by a Collison nebulizer on experimental day 98. The viral dose delivered (7.5×10^4 pfu) was calculated by standard procedures. Aerosol challenge was carried out within a plexiglass chamber, situated within a class III hood, contained within a biosafety level 3 laboratory equipped with

HEPA-filtered exhaust. Mice were monitored daily and the number of deaths recorded. The significance of differences in protection between groups receiving free and microencapsulated antigen was determined by the Fisher's exact test.

RESULTS AND DISCUSSION

Systemic Immunization With Microencapsulated VEE Virus Vaccine (29). In our initial series of experiments, we were interested in determining the effectiveness of microencapsulation on enhancing circulating antibody responses to VEE virus. Groups of BALB/c mice were immunized by SC injection of 50 μg free or microencapsulated formalin-fixed, inactivated TC-83 virus (29). The microspheres were composed of an equimolar ratio of DL-lactide and glycolide (50:50 DL-PLG), contained 0.81% (wt/wt) viral protein and were of a size range in which 90% of the internal volume was within microspheres of <17 μm in diameter. Plasma samples were collected prior to immunization and at 10 day intervals, and the levels of IgM, IgG and IgA anti-VEE virus activity were determined by ELISA. Following SC immunization of mice with microencapsulated TC-83, the level of circulating IgG anti-VEE virus antibody activity increased rapidly and peaked by day 20 (Fig. 1). The levels of antibody were higher than those induced with free TC-83 antigen. Following a second immunization on day 50, the level of circulating IgG antibody increased rapidly in mice given either free or microencapsulated antigen. The levels of antibody activity remained higher in mice given microencapsulated antigen than in animals given free antigen throughout the experiment. Only low or no IgM or IgA anti-VEE antibody activity was detected throughout the experiment.

To determine the optimal dose of microencapsulated VEE virus for induction of systemic responses, groups of BALB/c mice were given SC injections of 3.1, 6.2, 12.5, 25 or 50 μg (based on total viral protein) of formalin-fixed, inactivated VEE virus microencapsulated in 50:50 DL-PLG. A second set of mice were immunized with identical doses of non-microencapsulated (free) formalin-fixed, inactivated VEE virus. On day 50, all groups of mice were given a second SC injection of the same dose and form of VEE virus vaccine used for the primary immunization. IgG anti-VEE virus antibodies were detectable in all groups on day 30 following the initial immunization (Fig. 2). Mice given microencapsulated VEE virus had higher levels of IgG anti-VEE antibody activity than animals given free antigen at all doses tested. The level of antibody activity in mice given either microencapsulated or free antigen increased with increasing amounts of immunogen. In general, the microencapsulated VEE vaccine stimulated peak primary IgG anti-VEE titers 4- to 32-fold higher than dose-matched free vaccine recipients. Antibody responses following a secondary immunization on day 50 resulted in heightened antibody responses amongst all treatment groups. The responses were highest in mice given microencapsulated antigen. These results demonstrate that microencapsulated VEE virus was more effective than free virus antigen vaccine in inducing plasma IgG anti-VEE virus antibody responses.

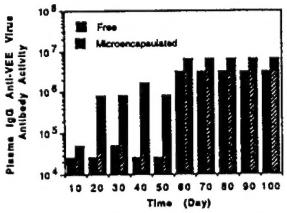

Fig. 1. Time course of the primary and secondary plasma IgG anti-VEE virus antibody responses in BALB/c mice immunized by subcutaneous injection of 50 μg of free or microencapsulated (50:50 DL-PLG; methylene chloride solvent) formalin-fixed, inactivated TC-83 virus on days 0 and 50.

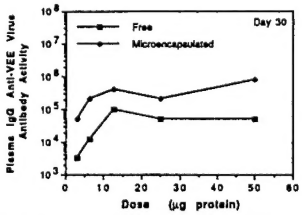

Fig. 2. Dose response of the primary plasma IgG anti-VEE virus antibody responses in BALB/c mice immunized by subcutaneous injection of the indicated dose of free or microencapsulated (50:50 DL-PLG; methylene chloride solvent) formalin-fixed, inactivated TC-83 virus on days 0 and 50.

In initial dose and time course studies, formalin-fixed, inactivated VEE virus was used as the immunogen. Therefore, it was of interest to determine if formalin-fixed virus was more immunogenic than untreated, inactivated virus following microencapsulation. Groups of mice were given SC injections of formalin-fixed or untreated, inactivated VEE virus microencapsulated in 50:50 DL-PLG microspheres on days 0 and 50. Higher levels of plasma IgG anti-VEE virus antibody activity were seen in mice immunized with formalin-fixed compared to non-formalin-fixed (untreated) microencapsulated VEE virus (Fig. 3). This difference in responses was seen after both the primary and secondary immunization and when either 12.5 or 50 μg of antigen was administered.

The microsphere vaccines used in the above described studies were prepared using methylene chloride as the processing solvent. Therefore, in order to determine if the solvent used for microencapsulation altered the immunogenicity of the VEE virus, microspheres containing formalin-fixed, inactivated VEE virus were prepared using either methylene chloride or ethyl acetate as solvent. These two preparations of 50:50 DL-PLG

microspheres contained similar amounts of antigen. Groups of mice were immunized by SC injection of 12.5 or 50 μg of microencapsulated VEE virus on days 0 and 50. The methylene chloride prepared microsphere vaccine induced higher plasma IgG anti-VEE virus antibody responses than the ethyl acetate processed microsphere vaccine in mice given 12.5 μg of microencapsulated antigen (Fig. 4). The level of antibody activity increased rapidly following the primary immunization with the methylene chloride microsphere vaccine. Responses were up to eight times higher (except on day 40) than those seen in mice given the ethyl acetate prepared microspheres. Ten days following the secondary immunization (experimental day 60), a similar level of antibody was seen in both groups of mice. The level of activity persisted in mice immunized with the methylene chloride microsphere vaccine, whereas an immediate drop in antibody activity was seen in mice given the ethyl acetate microsphere vaccine. When mice were immunized with higher amounts of antigen, i.e., 50 μg, both preparations of microsphere vaccine induced similar levels of antibody activity.

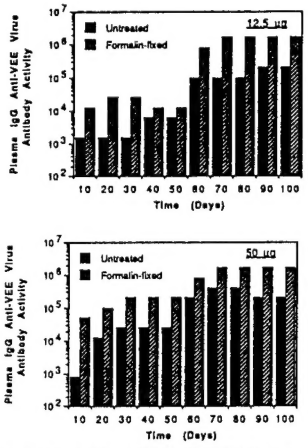

Fig. 3. Effect of formalin fixation of inactivated VEE virus on the immunogenicity of DL-PLG vaccines in BALB/c mice immunized by subcutaneous injection of 12.5 or 50 μg of microencapsulated formalin-fixed or untreated, inactivated TC-83 virus (50:50 DL-PLG; methylene chloride solvent) on days 0 and 50. Plasma samples were assayed for anti-VEE virus antibody activity by ELISA.

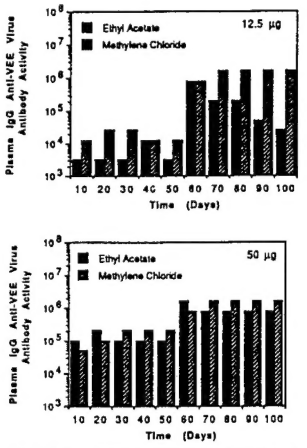

Fig. 4. Effect of solvent used for preparing microsphere vaccines on the induction of plasma IgG anti-VEE virus antibody responses in BALB/c mice immunized by subcutaneous injection of 12.5 or 50 μg of formalin-fixed, inactivated TC-83 virus in 50:50 DL-PLG microspheres prepared using either methylene chloride or ethyl acetate as the processing solvent on days 0 and 50.

Neutralization titers to VEE virus were determined in plasma samples obtained from groups of mice immunized on days 0 and 50 by SC injection of free or microencapsulated formalin-fixed, inactivated TC-83 virus. Mice given 50 μg of formalin-fixed VEE virus antigen encapsulated in microspheres prepared with methylene chloride mounted a primary neutralization response beginning at day 20 and an anamnestic responses after secondary immunization (Fig. 5). The peak neutralization titer was seen on day 80 and persisted through the termination of the study (day 100). The neutralization titers in this group were at least 16-fold higher than those obtained in mice immunized with free virus or virus microencapsulated using ethyl acetate as the processing solvent. Finally, mice immunized with free or microencapsulated non-formalin-fixed, inactivated VEE virus had no detectable circulating neutralization titers.

To assess the protective capabilities of the vaccine preparations, groups of mice were immunized with various doses of free or microencapsulated (prepared with the solvents methylene chloride or ethyl acetate) formalin-fixed, inactivated TC-83 virus. The immunized mice and a group of control, non-immunized animals were challenged by IP inoculation of the virulent V3000 strain of VEE virus on day 80 post the primary immunization. Mice immunized with 50 μg of TC-83 virus in ethyl acetate prepared DL-PLG microspheres were completely protected against challenge, whereas only 40% of mice given an equivalent dose of free antigen survived (Table 1). As little as 12.5 μg of antigen afforded 75% protection in mice receiving microencapsulated VEE virus, but only 30% in mice receiving the free VEE virus vaccine. All non-immunized control mice died following challenge. In a separate experiment, the protective capability of the methylene chloride processed DL-PLG microsphere vaccine compared to free TC-83 virus was evaluated. Mice immunized with a dose of 50 μg of free or microencapsulated antigen were completely protected against challenge. When a single dose of 5 μg was administered, mice receiving the microencapsulated vaccine were afforded 80% protection, whereas only 20% survival was seen with mice receiving free antigen. A 100% mortality was seen in the control, non-immunized group. Finally, mice receiving both a primary (day 0) and secondary (day 50) immunization with either free or microencapsulated TC-83 virus were protected against challenge with virulent V3000 virus on day 80 post secondary immunization, i.e., experimental day 130. Taken together, these results demonstrate that microencapsulated VEE virus was more effective than the free VEE virus vaccine in potentiating protective immune responses following systemic immunization. However, the relationship between circulating anti-VEE virus ELISA antibody activity, neutralization titers and protection against systemic challenge requires further investigation.

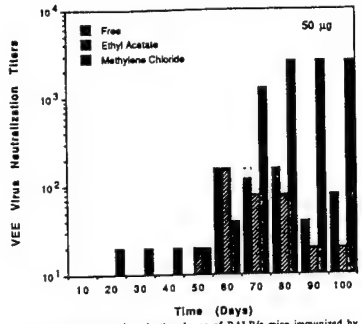

Fig. 5. Neutralization titers in the plasma of BALB/c mice immunized by subcutaneous injection of 50 µg of free or microencapsulated formalin-fixed, inactivated TC-83 virus (50:50 DL-PLG) prepared with methylene chloride or ethyl acetate solvent on days 0 and 50. The results are expressed as the reciprocol of the final dilution of serum that neutralized 80% of viral plaques.

Table 1. Effectiveness of microencapsulated VEE antigen in potentiating immune responses protective against challenge[a]

	Free VEE		Microencapsulated VEE		
Antigen Dose (µg)	Survivors/Total (Percent)	Mean Death Day	Microsphere Solvent	Survivors/Total (Percent)	Mean Death Day
50	4/10 (40%)	8.0	Ethyl acetate	10/10 (100%)	—[b]
25	4/10 (40%)	11.5		9/10 (90%)	9[c]
12.5	3/10 (30%)	8.7		6/8 (75%)	9
0	0/10 (0%)	9			
50	9/9 (100%)	—	Methylene chloride	10/10 (100%)	—
5	2/10 (20%)	12.5		8/10 (80%)	11.6[b]
0	0/10 (0%)10.7				

[a]Groups of mice were immunized on day 0 with free or microencapsulated formalin-fixed VEE. Mice were challenged i.p. with 15,000 pfu of the virulent V3000 strain of VEE virus on experimental day 80.
[b]Significant difference from free VEE virus group at $p < 0.01$.
[c]Significant difference from free VEE virus group at $p < 0.05$.

Mucosal Immunization With Microencapsulated VEE Virus Vaccine (30). In our second series of experiments, we were interested in determining the effectiveness of mucosal immunization with microencapsulated VEE virus in potentiating mucosal and systemic immune responses protective against aerosol challenge with virulent VEE virus (30). Groups of BALB/c mice were immunized by SC injection of 50 µg of formalin-fixed, inactivated TC-83 virus in microspheres (50:50 DL-PLG) prepared with ethyl acetate solvent on day 0 and boosted on day 30 by either oral or IT administration of 50 µg of the same microencapsulated virus vaccine. Samples of plasma collected prior to immunization and at 10 day intervals, and mucosal secretions collected on days 40, 50 and 60 were assessed for levels of IgM, IgG and IgA anti-VEE virus activity by ELISA. High plasma IgG anti-VEE antibody activity was seen by day 20 following SC immunization (Fig. 6). The levels of plasma IgG antibody activity increased in mice given a second immunization via the oral or IT route. Mice immunized by the IT route had the highest level of antibody activity which persisted through day 60. No or low plasma IgM or IgA responses were detected. When mucosal secretions from immunized animals were assessed for anti-VEE antibody activity, IT immunized mice had significantly higher levels of IgA and IgG antibody activity in BAW fluid than control animals 10 days after immunization (experimental day 40) (Table 2). Peak IgA and IgG anti-VEE antibody activity in BAW fluids and IgG antibody activity in fecal extracts were detected 20 days post IT immunization (experimental day 50). High levels of IgA and IgG antibody activity in BAW fluid were detected through day 60. Although the levels of antibody in BAW fluids and fecal extracts of orally immunized mice were higher than those seen in control animals, the differences were not significant. These results indicate the

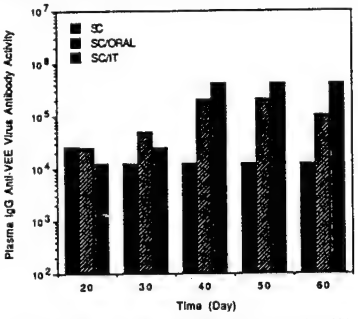

Fig. 6. Time course of the plasma IgG anti-VEE antibody response in BALB/c mice immunized by subcutaneous (SC) injection of 50 µg of formalin-fixed, inactivated TC-83 virus in microspheres (50:50 DL-PLG; ethyl acetate solvent) on day 0 and boosted on day 30 by either oral or intratracheal (IT) administration of 50 µg of the same microencapsulated virus vaccine.

Table 2. Time course of the mucosal immune responses induced in mice given a systemic followed by a mucosal immunization with VEE virus in ethyl acetate processed microspheres[a]

	Immunization Regimen	Anti-VEE Antibody Activity[b]					
		Day 40		Day 50		Day 60	
Specimen	(Day 0/Day 30)	IgG	IgA	IgG	IgA	IgG	IgA
Bronchial-Alveolar Wash	SC/None	420 ± 294	<10	190 ± 150	<10	35 ± 5	<10
	SC/Oral	384 ± 64	26 ± 14	424 ± 241	40 ± 30	384 ± 228	<10
	SC/IT	3,072* ± 868	88* ± 20	4,608* ± 1,649	176* ± 63	2,245* ± 1,090	40* ± 23
Saliva	SC/None	20 ± 0	20 ± 0	20 ± 0	20 ± 0	25 ± 5	20 ± 0
	SC/Oral	20 ± 0	20 ± 0	20 ± 0	20 ± 0	20 ± 0	20 ± 0
	SC/IT	20 ± 0	24 ± 4	276 ± 251	20 ± 0	20 ± 0	20 ± 0
Gut Wash	SC/None	33 ± 7	<5	8 ± 1	28 ± 18	170 ± 67	<5
	SC/Oral	74 ± 25	<5	40 ± 30	9 ± 3	36 ± 31	<5
	SC/IT	584* ± 207	<5	200 ± 112	39 ± 17	36 ± 31	<5
Fecal Extract	SC/None	50 ± 0	27 ± 12	90 ± 29	510 ± 309	100 ± 35	925 ± 394
	SC/Oral	162 ± 38	162 ± 80	970 ± 291	820 ± 347	1,640 ± 1,194	2,080 ± 480
	SC/IT	380 ± 174	120 ± 34	4,000* ± 1,073	600 ± 253	2,612 ± 1,430	900 ± 232

[a]Groups of mice (15/group) were immunized by subcutaneous (SC) administration of 50 µg of formalin-fixed, inactivated TC-83 virus in ethyl acetate processed microspheres. Secretions were collected from mice (5/group) on days 40, 50 and 60, processed and assayed for antibody activity by ELISA. No IgM antibody activity was detected.
[b]Values are the mean ± SEM of the reciprocol of the end point dilution. Asterisk (*) indicates values which are significantly different ($p \leq 0.05$) from the control SC/None group.

effectiveness of IT immunization in inducing both systemic and mucosal immune responses.

In order to determine if the solvent used to prepare the microencapsulated antigen had an effect on the ability of the vaccine to potentiate an immune response, additional groups of mice were immunized with microspheres containing VEE which were prepared with the solvent methylene chloride. In this experiment, a group of mice was included that received both a primary and secondary immunization by the IT route. By day 20 following immunization, mice given a SC immunization had good plasma IgG anti-VEE antibody responses (Fig. 7). The level of antibody activity seen was similar to that observed in mice immunized with the microsphere vaccine prepared with the solvent ethyl acetate (Fig. 6). A lower plasma IgG anti-VEE response was seen in mice immunized by the IT route. A secondary immunization by the SC or IT route resulted in an augmented response by day 40 (Fig. 7), whereas an enhancement in the plasma IgG anti-VEE response was seen on day 50 in orally immunized mice.

BAW fluid obtained from mice 20 days (day 50) after immunization by the IT route had higher levels of IgG and IgA anti-VEE antibody activity that animals immunized by the SC route (Table 3). Mice immunized only by the IT route had significantly higher levels of IgA in gut fluids. In general, responses seen with the ethyl acetate processed microspheres (Table 2) were higher than those seen with the microspheres prepared with methylene chloride.

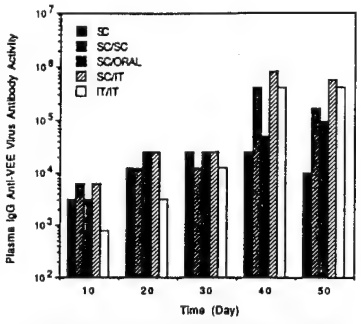

Fig. 7. Time course of the plasma IgG anti-VEE antibody response in BALB/c mice immunized by the administration of 50 µg of formalin-fixed, inactivated TC-83 virus in microspheres by the subcutaneous (SC) (50:50 DL-PLG; methylene chloride solvent) or the intratracheal (IT) (50:50 DL-PLG; methylene chloride solvent) route on day 0 and boosted on day 30 by subcutaneous (SC), oral or intratracheal (IT) administration of 50 µg of the same microencapsulated virus vaccine.

Table 3. Induction of mucosal immune responses in mice given a systemic followed by a mucosal immunization with microencapsulated VEE virus prepared with methylene chloride.

Immunization Regimen[a] (day 0/day 30)	Anti-VEE Antibody Activity[b]							
	Bronchial Alveolar Wash		Saliva		Gut Wash		Fecal Extract	
	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA
SC/None	308 ± 243	134 ± 127	16 ± 16	8 ± 8	132 ± 127	8 ± 3	50 ± 22	<50
SC/SC	600 ± 248	<10	70* ± 33	<20	121 ± 39	3 ± 0	63 ± 24	<50
SC/Oral	304 ± 244	134 ± 126	20 ± 0	4 ± 4	13* ± 7	6 ± 1	80 ± 80	<50
SC/IT	2,304* ± 256	456 ± 213	20 ± 0	4 ± 4	96 ± 86	5 ± 0	60 ± 40	<50
IT/IT	1,216* ± 384	480 ± 221	<20	20 ± 1	262 ± 117	36* ± 4	50 ± 0	810* ± 329

[a]Groups of mice (5/group) were immunized by either subcutaneous (SC) or intratracheal (IT) administration of 50 µg formalin-fixed, inactivated TC-83 virus in methylene chloride processed microspheres. Secretions were collected on day 50 and processed and assayed for antibody activity by ELISA. No IgM antibody activity was detected.
[b]Values are the mean ± SEM of the reciprocal of the end point dilution. Asterisk (*) indicates values which are significantly different ($p \leq 0.05$) from the control SC/None group.

In order to determine the effectiveness of the immunizations on the induction of protective immune responses, groups of mice immunized via the SC, oral or IT route with microencapsulated VEE and a group of nonimmunized controls were challenged 68 days post secondary immunization (day 98) with an infectious aerosol of V3000 virus generated by a Collison nebulizer (Table 4). Although protection was seen in all groups of immunized mice, only animals immunized by the IT route showed 100% protection. These results suggest that the mucosal response induced by IT immunization provided an additional protection to that seen as a result of a systemic immune response. Further studies will be required to establish the relationship between mucosal versus systemic immune responses and protection against aerosol challenge.

Table 4. Effectiveness of microencapsulated VEE virus vaccine in protentiating immune responses protective against aerosol challenge.[a]

Immunization Regimen (Day 0/Day 30)	Survivors/Total (Percent)	Mean Death Day
SC/SC	8/9 (89%)	11
SC/ORAL	9/10 (90%)	10
SC/IT	10/10 (100%)	–
IT/IT	9/9 (100%)	–
Controls		
Nonimmunized	0/10	11.6

[a]Groups of mice were immunized on days 0 and 30 with 50 µg VEE virus in methylene chloride processed microspheres (50:50 DL-PLG; Batch G320-140-00) and challenged on day 98 with an infectious aerosol of V3000 virus (7.5×10^4 pfu) generated by a Collison nebulizer.

Pulse Release Studies With Microencapsulated VEE Virus Vaccine. Previous studies have shown that the rate of antigen release from microspheres will vary based on the lactide to glycolide composition. Therefore, our next series of experiments were designed to determine the kinetics of the immune response induced after systemic immunization with microspheres composed of different proportions of lactide to glycolide and containing VEE virus antigen. Groups of BALB/c mice were given SC immunizations with different doses of the various preparations of microencapsulated VEE virus antigen and the kinetics of the immune responses were assessed throughout a 300 day experiment (Fig. 8). Mice immunized with 50 µg VEE virus in 50:50 DL-PLG microspheres showed a rapid response which peaked at ~day 60 and began to drop at ~day 200 (Fig. 8A). The same dose of antigen in 85:25 DL-PLG microspheres induced a response which increased slowly and peaked at ~day 180 after immunization. Microspheres prepared with lactide only induced the lowest response which peaked on ~day 160. Finally, mice given a combination of 50:50 and 100:0 DL-PLG microspheres, each containing 25 µg antigen (50 µg total dose) had the highest initial response which decreased gradually, but persisted through day 300. When 25 µg of vaccine antigen was given in the various microsphere preparations, a response pattern was seen which was similar, but lower than that seen with a 50 µg dose of the same vaccine preparations (Fig. 8B). The peak response in mice given the 50:50 DL-PLG microspheres occurred on ~day 40. Animals given a mixture of all three microsphere preparations (at a 25 µg dose of each) had the highest response which persisted at close to maximum level throughout the 300 day experiment. As little as 12.5 µg of antigen in 50:50 DL-PLG microspheres was still effective in inducing an immune response which peaked on ~day 30 and began to drop on ~day 200 (Fig. 8C). However, microspheres containing 12.5 µg VEE virus and composed of either 85:25 or 100:0 DL-PLG induced only a low response. When mice were immunized with 25 µg of antigen in a mixture of 50:50 and 100:0 DL-PLG microspheres (12.5 µg of antigen each), the response induced (Fig. 8C) was initially higher than that seen with a dose of 50:50 DL-

PLG microspheres containing 25 μg antigen (Fig. 8B). The response pattern in these groups of mice were similar after the initial early peak. Taken together, these results suggest that: 1) low levels of 50:50 DP-PLG microspheres containing VEE virus antigen are effective in inducing responses; 2) microspheres composed of 85:25 or 100:0 DL-PLG and containing VEE virus antigen induce a late response; and 3) combinations of microspheres composed of different proportions of lactide to glycolide induce a higher initial response which persists for a longer period than any of the individual microsphere preparations.

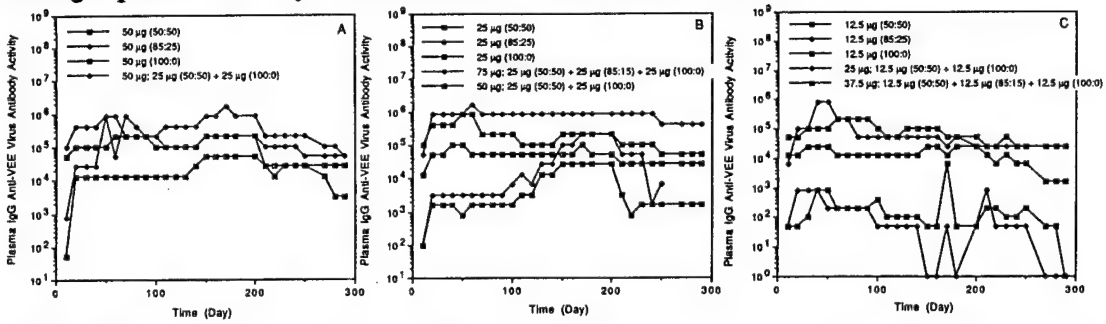

Fig. 8. Effect of the lactide to glycolide composition of microspheres on the time course of the immune response induced following systemic administration of microencapsulated VEE virus antigen.

Systemic Immunization With Microencapsulated Protective Antigen of *Bacillus anthracis* In other studies, we have also looked at the effectiveness of microencapsulating the PA of *B. anthracis* in inducing potentiated immune responses. In one series of experiments, groups of mice were immunized with 12.5 or 50 μg PA alone, in microspheres or in alhydrogel by the SC route on days 0 and 50. Samples of plasma were collected prior to immunization and at 10 day intervals throughout the experiment and assayed for anti-PA antibody activity by ELISA. Mice immunized with 12.5 μg of microencapsulated PA had higher levels of anti-PA antibody activity after the primary immunization than mice given PA alone or in alhydrogel (Fig. 9). Higher responses were also seen after the second immunization. However, when the responses began to decrease, the level of antibody activity in mice immunized with PA in microspheres or in alhydrogel were similar. When a higher amount of antigen was given (50 μg), the microsphere and the alhydrogel vaccines induced primary responses which were higher than that obtained with PA alone (Fig. 9). After the secondary immunization, highest responses were seen in mice given microencapsulated PA.

In our studies with microencapsulated VEE virus vaccines, some difference was seen in the immune response induced with microspheres prepared using methylene chloride or ethyl acetate as solvent. Therefore, in a second series of experiments, groups of mice were immunized with microspheres containing PA which were prepared with the solvent methylene chloride or ethyl acetate (Fig. 10). Following the primary immunization, all three preparations of microencapsulated PA (especially those prepared with methylene chloride) potentiated immune responses to PA. All three microsphere vaccine preparations induced similar secondary responses in mice given 12.5 μg of antigen, whereas one of the two methylene chloride vaccine preparations tested induced the highest secondary response when 50 μg of PA was used as the immunizing dose. In this study, the core load of antigen in the microsphere preparations differed and further studies will be required to establish if this had an effect on resulting immune response.

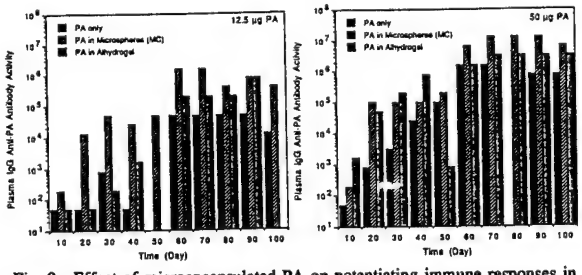

Fig. 9. Effect of microencapsulated PA on potentiating immune responses in BALB/c mice following systemic immunization.

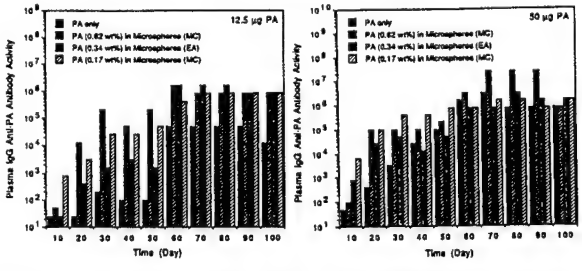

Fig. 10. Effect of solvent used to prepare microencapsulated PA on their ability to potentiate immune responses in BALB/c mice.

In order to determine the efficacy of the microencapsulated PA vaccine, a separate series of experiments was performed in Hartley guinea pigs at USAMRIID by Dr. Arthur M. Friedlander. Groups of animals were immunized at 0 and 7 weeks with various PA vaccine preparations (Table 5). The animals were challenged at 13 weeks with ~200 aerosol LD_{50}s of Ames spores. Prior to challenge, the plasma anti-PA titers were determined in each group of guinea pigs. Approximately 70% of the animals immunized with microencapsulated PA or MPL + PA were protected from aerosol challenge compared to only 13% of animals immunized with MDPH-PA.

Interestingly, animals immunized with microencapsulated PA had 10-fold lower anti-PA titer than that seen in guinea pigs given MPL + PA. The anti-PA titer in animals given MDPH-PA was only about half that seen in animals given MPL + PA. Furthermore, animal immunized with alhydrogel + PA had a high anti-PA titer, but only 19% survived. These results demonstrate that the microencapsulated PA vaccine was highly effective in inducing protection against aerosol challenge with Ames spores. Furthermore, the results suggest that the anti-PA titer does not directly correlate with protection. It is possible that the assay being used to assess antibody activity is not accurately reflecting the level of antibodies to protective epitopes of PA. It is also possible that immune mechanisms other than humoral are important in protection against *B. anthracis*.

Table 5. Effectiveness of a microencapsulated PA vaccine in inducing protective immune responses in guinea pigs.

Vaccine[a]	Survived/Total	% Survival	Anti-PA Titers
EA-microencapsulated PA	6/8	75	316
MC-microencapsulated PA	5/8	63	366
MPL + PA	11/15[b]	73	3,689
Alhydrogel + PA	3/16	19	2,154
MDPH-PA	2/15	13	1,711
PBS only	0/8	0	<10

[a]Guinea pigs (equal numbers of male and females in each group) were immunized at 0 and 7 weeks, then challenged at 13 weeks with 120-230 aerosol LD_{50}s of Ames spores. EA, ethyl acetate; MC, methylene chloride; MPL, monophosphoryl lipid A; MDPH-PA, licensed human anthrax vaccine consisting of aluminum hydroxide-adsorbed crude PA.
[b]Significantly different from MDPH-PA ($p < 0.05$).

DISCUSSION AND CONCLUSIONS

Considerable interest during the past several years has been directed towards the development of efficacious and effective means of inducing protective immune responses against airborne infectious agents. Evidence is accumulating which indicates that this will require that the vaccine antigen be incorporated into an improved adjuvant and/or delivery system and that the vaccine will induce both enhanced levels of circulating antibody responses, as well as S-IgA antibody responses in the secretions which bathe the upper respiratory tract. Results obtained in this contract effort indicate that this is possible through the use of a microsphere delivery system.

During this contract effort, the processing conditions for effective encapsulation of ^{60}Co-inactivated and formalin-fixed whole VEE virus or of PA of *B. anthracis* vaccine in microspheres formulated with the biodegradable and biocompatible copolymer DL-PLG were developed. These conditions were derived for two processing solvents, methylene chloride and ethyl acetate, each of which have been used for the encapsulation of at least two other inactivated whole virus-based vaccines and several microbial protein-based vaccines. These prototype vaccine microspheres contain between 0.5 and 1.0% vaccine by weight, show a uniform surface morphology and minimal vaccine release through imperfections in the microsphere polymer wall, and approach the ideal size distribution of 1 to 10 μm in diameter. Thus, using two different process solvents, the VEE virus and the PA of *B. anthracis* vaccines have been encapsulated in microspheres with physical characteristics which have been characterized as providing maximal potentiation of the humoral response and adsorption into MALT.

In vivo evaluation of the immunogenicity of the microspheres vaccines in mice following SC injection confirmed that significant enhancement of the circulating anti-VEE virus and anti-PA antibody responses was induced by the microsphere vaccines. The circulating antibody responses were predominantly of the IgG isotype. Furthermore, the responses induced with the microsphere vaccines were higher than those seen with an antigen only vaccine over a broad dose range. These results demonstrate the effectiveness of microencapsulated vaccines in potentiating a circulating IgG antibody response, the isotype of importance in effecting protection against pathogens which enter the blood circulation.

In our studies with the VEE virus vaccines, it was shown that microencapsulated, formalin-fixed VEE virus induced higher plasma IgG antibody responses than microencapsulated, non-formalin-fixed virus over a broad dose range. These results provide evidence that formalin-fixation prevented degradation of antigenic epitopes due to the microencapsulation procedure. Furthermore, it is possible that the formalin-fixation stabilizes antigenic epitopes for more effective presentation to the host immune elements. The solvent used to prepare the microencapsulated VEE virus also effected the immunogenicity of the vaccine. The vaccine prepared with methylene chloride compared to the vaccine prepared with ethyl acetate induced higher and more persistent ELISA-reactive antibody levels with a dose of 12.5 μg and neutralizing titers with a dose of 50 μg. However, this relationship was not true at other doses. Finally, when the effectiveness of the immune responses induced with the different vaccine preparations was assessed in terms of there ability to protect the host against systemic challenge, the greatest protection was seen in mice immunized with microencapsulated VEE virus. Taken together, our results demonstrate that microencapsulated VEE virus was more effective than the free VEE virus vaccine in potentiating protective immune responses following systemic immunization. However, the relationship between circulating anti-VEE virus ELISA antibody activity, neutralization titers and protection against systemic challenge is not clear and further studies will be required. VEE virus is only one member of the alphaviruses

which is capable of producing severe and fatal encephalitic disease in humans and horses. Eastern and Western equine encephalitis (EEE and WEE, respectively) virus are endemic in the United States. Therefore, based on the present results, it would be beneficial to establish the effectiveness of microencapsulating these viruses for use as vaccines in inducing protective immunity. However, important concerns regarding the development of vaccines against equine encephalomyelitis viruses are the issues of cross-protective immunity and immune interference, since conflicting results exist. Thus, questions regarding these issues should be addressed in future studies.

Immunization by the mucosal route, i.e., via the oral and especially the IT routes, with microencapsulated VEE virus vaccine was shown to potentiate not only a plasma IgG response, but it was also effective in inducing a mucosal response. In this regard, mice primed by the systemic route followed by immunization by the IT route or immunized by the IT route only had elevated IgG and IgA antibody levels in their bronchial-alveolar wash fluid. Furthermore, all mice immunized by the IT route were protected against aerosol challenge with infectious VEE virus. These results demonstrated that the mucosal responses induced by IT immunization provided an additional protection to that seen as a result of a systemic immune response only. However, further studies will be required to establish the relationship between mucosal versus systemic immune responses and protection against aerosol challenge. Since aerosol challenge by infectious agents will likely involve nasal surface, it would be of interest to determine if immunization via the intranasal (IN) route would effect protective responses. Based on accumulating evidence, IN immunization induces mucosal, especially in the upper respiratory tract, as well as systemic responses depending on the nature of the vaccine and should prove to be an effective means for protecting the host against inhaled infectious agents. This route of immunization has several advantages, including effectiveness, safety, decreased side effects, and the potential for almost unlimited number and frequency of convenient boostings. Furthermore, the dose of antigen required for IN immunization is likely to be less than that needed for immunization by other mucosal routes.

Our studies with the PA of *B. anthracis* demonstrated that protein antigens from microbial pathogen can be microencapsulated and when used for immunization, result in the induction of augmented systemic immune responses which are protective against aerosol challenge with Ames spores. These results support the use of the microsphere technology in testing the effectiveness of microencapsulated vaccines against other infectious agents, such as *Yersinia pestis*. In our studies with VEE virus and PA vaccines, a clear relationship between ELISA-antibody activity, neutralization titers and protection were not discerned. Since it is likely that cellular immune elements also play a role in protection, future studies should establish the relationship between the humoral and cellular immune element in protection.

In the development of a vaccine, parameters to consider are the ease of delivery, the vaccine dose required, and the frequency of boosting. The use of a pulse-release microsphere vaccine containing VEE virus was shown to induce a prolonged, high titer plasma antibody response. Further studies will be required to determine how effective the response is in protection and how effective this approach will be with other vaccine antigens. Although microencapsulated vaccines did result in elevated immune responses, numerous investigations including studies with PA of *B. anthracis* have demonstrated that adjuvants such as monophosphoryl lipid A and the B subunit of cholera toxin may be very useful vaccine supplement for further potentiating systemic and mucosal responses. The potential usefulness of these adjuvants in the development of vaccines to protect against air-borne infectious agents requires further investigation.

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